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Research Triangle Park, North Carolina

Optimization of the Sliced Testis Steroidogenesis Assay

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RTI International

August 2003

This study was performed under a subcontract to Battelle Laboratories, Columbus, Ohio for prime contract No. 68-W-01-023 for the U.S. Environmental Protection Agency

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Optimization of the Sliced Testis Assay

This was Implemented in Two Phases

Basic
Sliced Testis Assay

- At first timepoint –designated baseline – media is removed and discarded
- Fresh media is added and an aliquot is collected
- Half of the samples are challenged with a stimulant, such as hCG
- Aliquots of media are collected at 1, 2, 3 and 4 hours post-challenge
- Medium samples are analyzed for testosterone concentration in a RIA assay
- Medium samples are analyzed for Lactate Dehydrogenase (LDH) as a measure of cell viability in a spectrophotometric assay

Technical Flow Illustration of Sliced Testis Assay

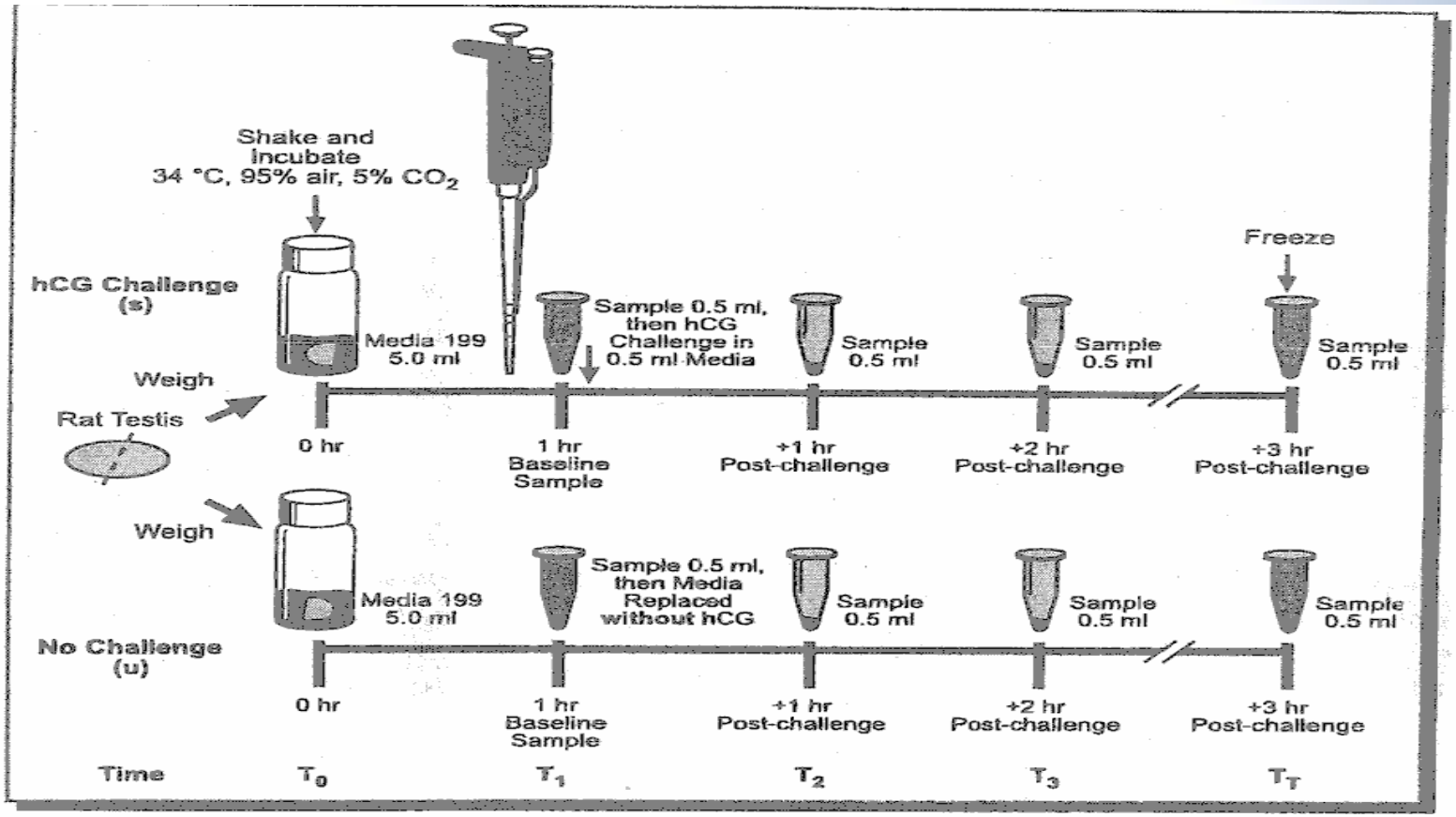

Figure 2. Technical Flow Illustration of the Sliced Testis Steroidogenesis Assay

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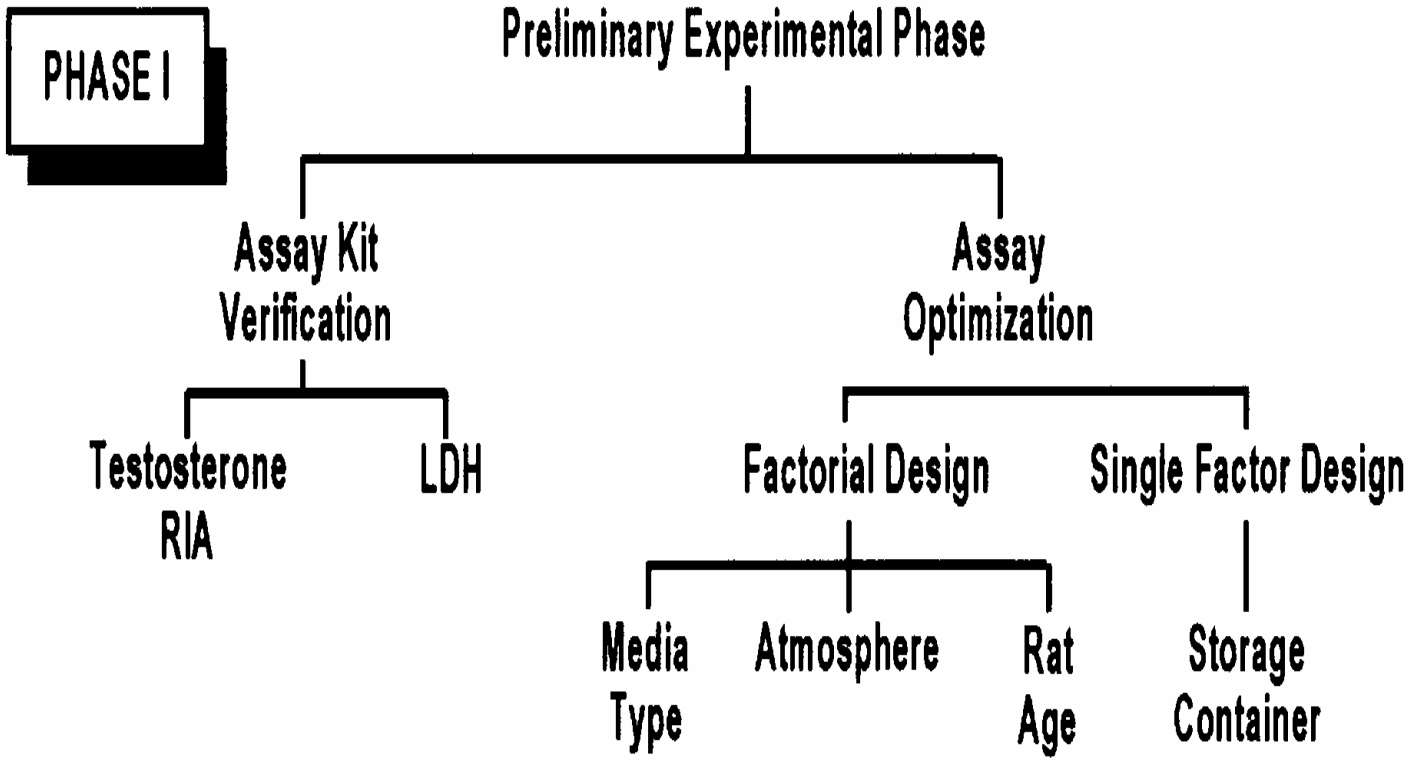
Phase I Design



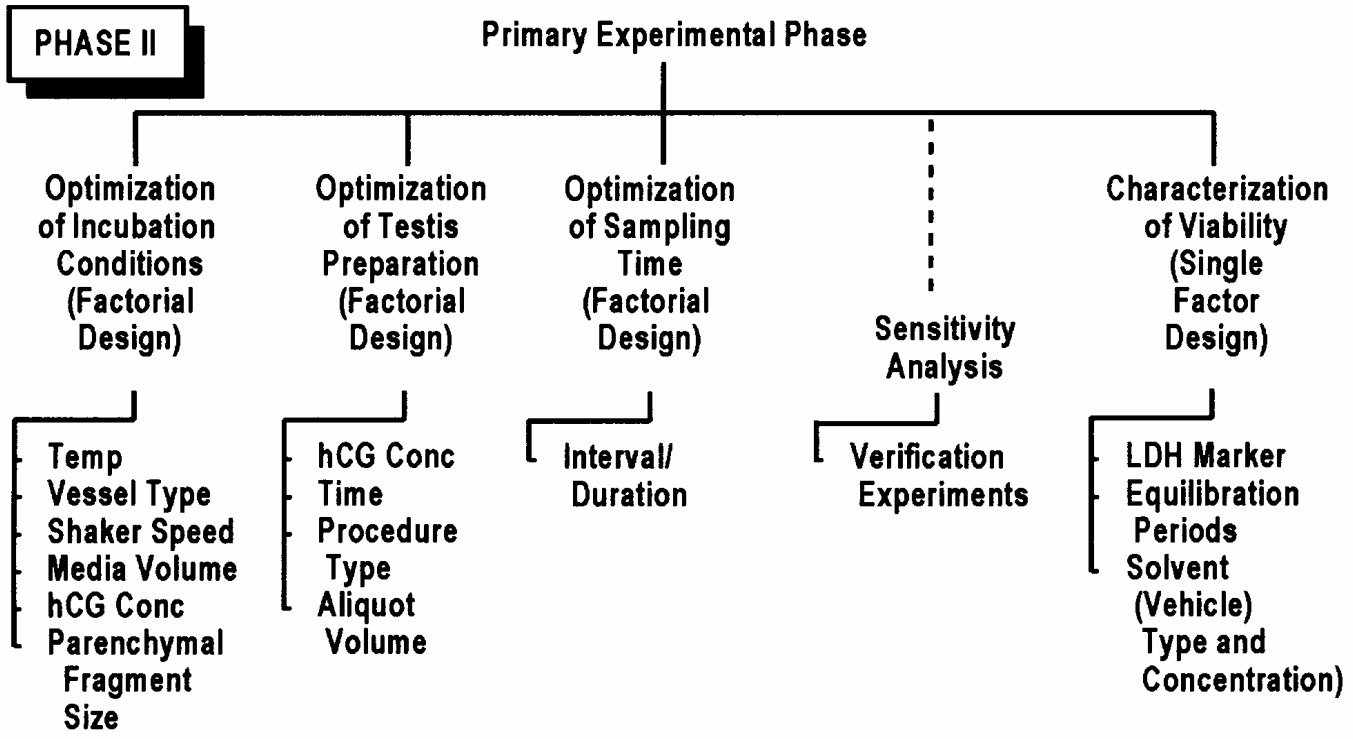
Results of Phase I Optimizations

- Medium-199 without phenol red
- Gaseous atmosphere of 5% CO_2 / 95% O_2
- Rats that were 11 weeks of age showed results similar to those that were 15 weeks of age, therefore rats 11-15 weeks can be used in the assay; 22 week old rats exhibit reduced testosterone production

Phase II: Primary Experimental Phase

- Factors that may affect assay performance were tested
- These factors were divided into four sections where each section was composed of factors that might produce interactions

Phase II Design



Phase II

- To be tested:
  - Incubation Conditions
    - Incubation Temperature
    - Incubation Vessel Type
    - Incubation Shaker Speed
    - Incubation Media Volume
    - hCG Concentrations
  - Testes Preparation Conditions
    - Testicular Fragment Size
    - Time Delay before starting the assay preparation
    - Organ Preparation Technique
    - Sample Aliquot Volume

Table 4. Summary of Experimental Incubation Factors for Optimization

Factor Identification	Units	Factor Name	Experimental Levels					Coded Experimental Levels				
			1	2	3	4	5	1	2	3	4	5
Incubation Temperature	°C	X1	--	34*	--	37	--	--	-1	--	+1	--
Incubation Vessel Type	NA[a]	X2	scintillation vial*	test tube	--	--	--	-1	+1	--	--	--
Incubation Shaker Speed	NA	X3	--	none	low	high *	--	--	-1	0	+1	--
Incubation Media Volume	ml	X4	--	2.5	5*	10	--	--	-1	0	+1	--
hCG Concentration	IU/ml	X5	0.001	0.01	0.1*	1	10	-2	-1	0	+1	+2
Fragment Size	mg	X6	25	50	125	250*	--	-0.8	-0.6	0	+1	--

* Prototypical value.

[a] NA - not applicable.

ANOVA results for PROC RSREG with the Baseline Concentration Removed from Model

hCG	Vessel Type	Error df	Time	R^2	hCG Conc.	Incub. Vol.	Shaker Speed	Frag. Size	Incub. Temp.	RMSE
No	scintillation vial	24	T1	77	NA	***	***	**	**	0.532
			T2	72	NA	***	**	*	*	0.600
			T3	72	NA	***	***	*		0.625
			T4	69	NA	***	**	*		0.653
	test tube	14	T1	71	NA		***		*	0.390
			T2	76	NA	*	***		*	0.279
			T3	76	NA	**	***	**	*	0.316
			T4	82	NA	***	***	**	**	0.300
Yes	scintillation vial	16	T1	91		***	***	***		0.455
			T2	88	*	***	***	***	*	0.552
			T3	85		***	*	***	*	0.681
			T4	85		***	*	***	*	0.693
	test tube	12	T1	93	***	***	***	***	***	0.278
			T2	92	**	***	***	***	***	0.315
			T3	94	***	***	***	***	***	0.288
			T4	87	*	***	***	**	***	0.447

Note: R^2 is the percentage of variation accounted for by the model.
RMSE = square root of the residual (error) mean square

Predicted Values for Specified Optimum Factor Combinations for Models with Baseline Concentration Removed

hCG	Vessel Type	Time	Predicted Log(Testosterone Concentration-ng/g))								Media Volume (mL)	hCG Concentration (IU/mL)	Fragment Size (mg)	Temp (°C)	Shaker Speed Code	Shaker Speed (rpm)
			T1		T2		T3		T4							
No	Scintillation vial	Optimal	3.243	(0.228)	3.257	(0.258)	3.687	(0.268)	3.758	(0.280)	5.0		175.0	36.0	0.0	low
No	Scintillation vial	Optimal	3.137	(0.288)	3.012	(0.326)	3.082	(0.339)	3.150	(0.354)	5.0		175.0	36.0	1.0	high
Yes	Scintillation vial	Optimal	4.586	(0.261)	4.888	(0.316)	5.609	(0.390)	5.914	(0.397)	2.5	0.100	175.0	36.0	0.0	low
Yes	Scintillation vial	Optimal	5.178	(0.308)	5.700	(0.374)	6.283	(0.461)	6.468	(0.469)	2.5	0.100	175.0	36.0	1.0	high

*Values in parentheses are standard errors

Conclusions from Incubation Factors Experiments

- Optimal temperature for incubation was approximately 36ºC
- Optimal vessel for incubation was the scintillation vial
- Optimal Shaker speed was between 135 and 200 rpm, approximately 175 rpm
- Optimal media volume was approximately 4-5 mL
- Optimal hCG concentration for stimulation was 0.08 to 0.1 IU/mL

Table 6. Summary of Experimental Testis Preparation Factors for Optimization

Factor Identification	Units	Factor	Experimental Levels			Coded Experimental Levels		
			1	2	3	1	2	3
hCG Concentration	IU/ml	X5	0.01	0.1*	1	-1	0	+1
Time Delay	hr	X7	0.5	1*	2	-1	0	+1
Organ Preparation Technique	NA[a]	X8	Cold buffered saline	Warm buffered saline	Cold media*	-1	0	+1
Sample Aliquot Volume	ml	X9	0.125	0.25	0.5*	-1	0	+1

*Prototypical value.

[a] NA - not applicable.

ANOVA results for PROC RSREG with the Baseline Concentration Removed from Model

hCG	Organ Preparation Solution	Error df	Timepoint	R^2	hCG Concentration (IU/mL)	Time Delay (Hr.)	Aliquot Volume (mL)	RMSE
no	Cold Buffered Saline	13	T1	89	NA	***		0.261
			T2	80	NA	***		0.374
			T3	80	NA	***		0.342
			T4	81	NA	***		0.334
	Cold Media	12	T1	87	NA	***		0.201
			T2	75	NA	***		0.333
			T3	89	NA	***		0.153
			T4	92	NA	***	*	0.139
	Warm Buffered Saline	12	T1	66	NA	***		0.438
			T2	63	NA	***		0.471
			T3	55	NA	**		0.510
			T4	48	NA	*		0.646
yes	Cold Buffered Saline	7	T1	89		***		0.292
			T2	77		**		0.378
			T3	74		*		0.421
			T4	69				0.449
	Cold Media	7	T1	97		***		0.109
			T2	86		**		0.304
			T3	73		*		0.590
			T4	69				0.698
	Warm Buffered Saline	7	T1	84	**			0.450
			T2	87		**		0.455
			T3	67				0.664
			T4	80	*	*		0.578

ote:
R^2 is the percentage of variation accounted for by the model.
RMSE = square root of the residual (error) mean square

Predicted values for Specified Optimum Factor Combinations for Models with Baseline Concentration Removed

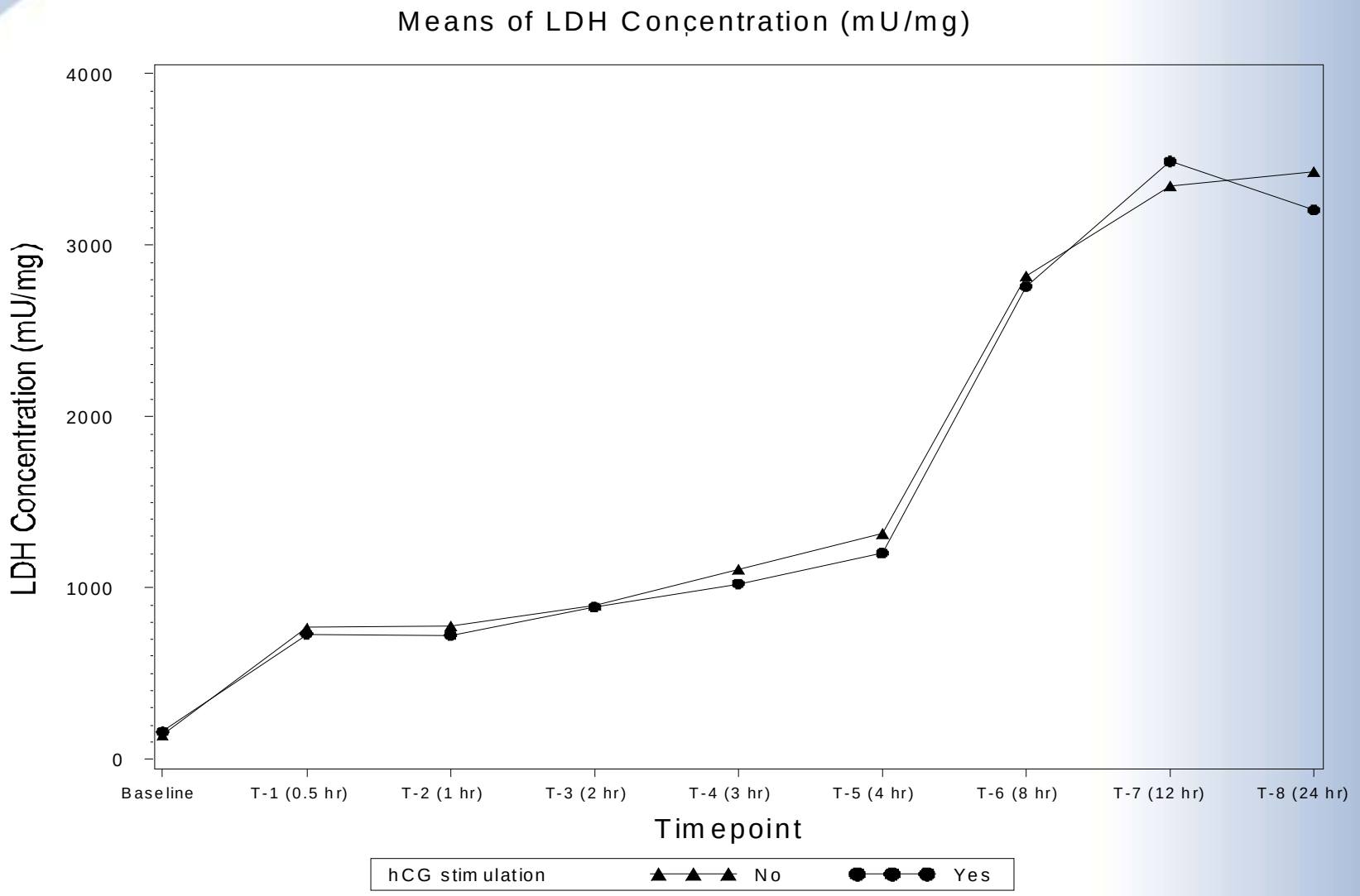
hCG	Organ Prep Technique		Predicted Log (Testosterone Concentration- ng/g))*				Sample Volume (mL)	hCG Concentration (IU/mL)	Time Delay (hr)
			T1	T2	T3	T4			
No	Cold Buffered	Optimal	2.815 (0.157)	2.973 (0.225)	3.227 (0.205)	3.317 (0.200)	0.5		1.0
No	Cold Buffered	Optimal	3.872 (0.169)	4.048 (0.243)	4.242 (0.222)	4.359 (0.217)	0.5		0.5
Yes	Cold Buffered	Optimal	3.344 (0.218)	4.145 (0.282)	4.482 (0.314)	4.731 (0.334)	0.5	0.100	1.0
Yes	Cold Buffered	Optimal	4.337 (0.236)	5.154 (0.305)	5.535 (0.339)	5.676 (0.361)	0.5	0.100	0.5

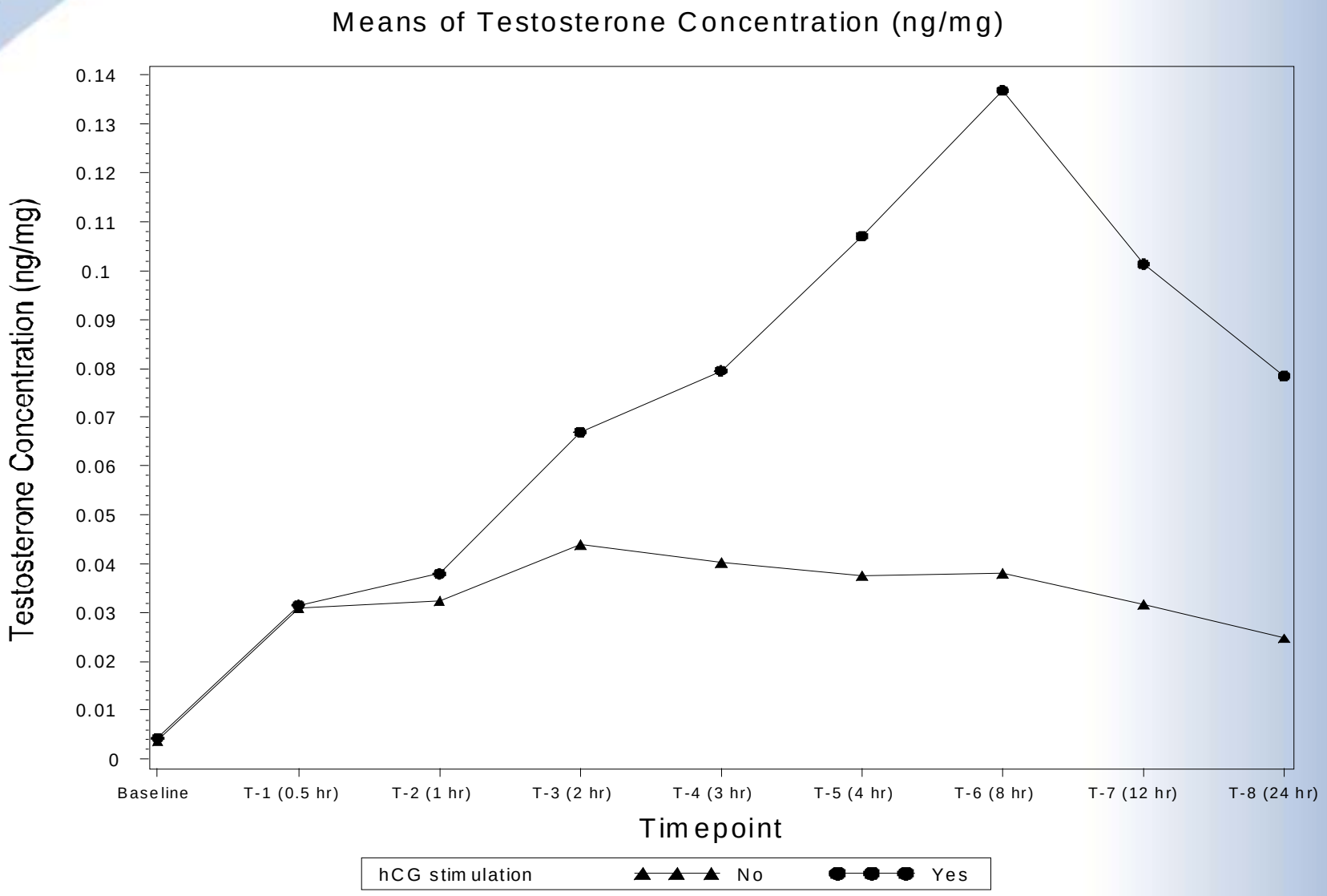
*Values in parentheses are standard errors

Conclusions from Testes Preparation Experiments

- Optimal fragment size varied from 100-200 mg with stimulated versus non-stimulated assays
- Time delay before the start of the incubation should be no more than 1 hour from the time of testicular tissue removal from the male
- The solution that the testes are collected in may be either cold DPBS or cold M-199
- The sample aliquot size removed for testing should be around 0.5 mL

Coefficient of Variation Values (%) for Testosterone Concentrations by Sampling Timepoints in the Optimized Assay									
hCG	Baseline	0.5 hr	1 hr	2 hr	3 hr	4 hr	8 hr	12 hr	24 hr
No	18.9	11.0	6.2	22.1	12.2	14.89	10.52	15.5	19.4
Yes	18.6	15.6	16.4	22.0	38.1	16.1	36.6	28.8	24.5


Means of LDH Concentration (mU/mg)
LDH Concentration (mU/mg)
4000
3000
2000
1000
0
Baseline
T-1 (0.5 hr)
T-2 (1 hr)
T-3 (2 hr)
T-4 (3 hr)
T-5 (4 hr)
T-6 (8 hr)
T-7 (12 hr)
T-8 (24 hr)
Timepoint
hCG stimulation
No
Yes



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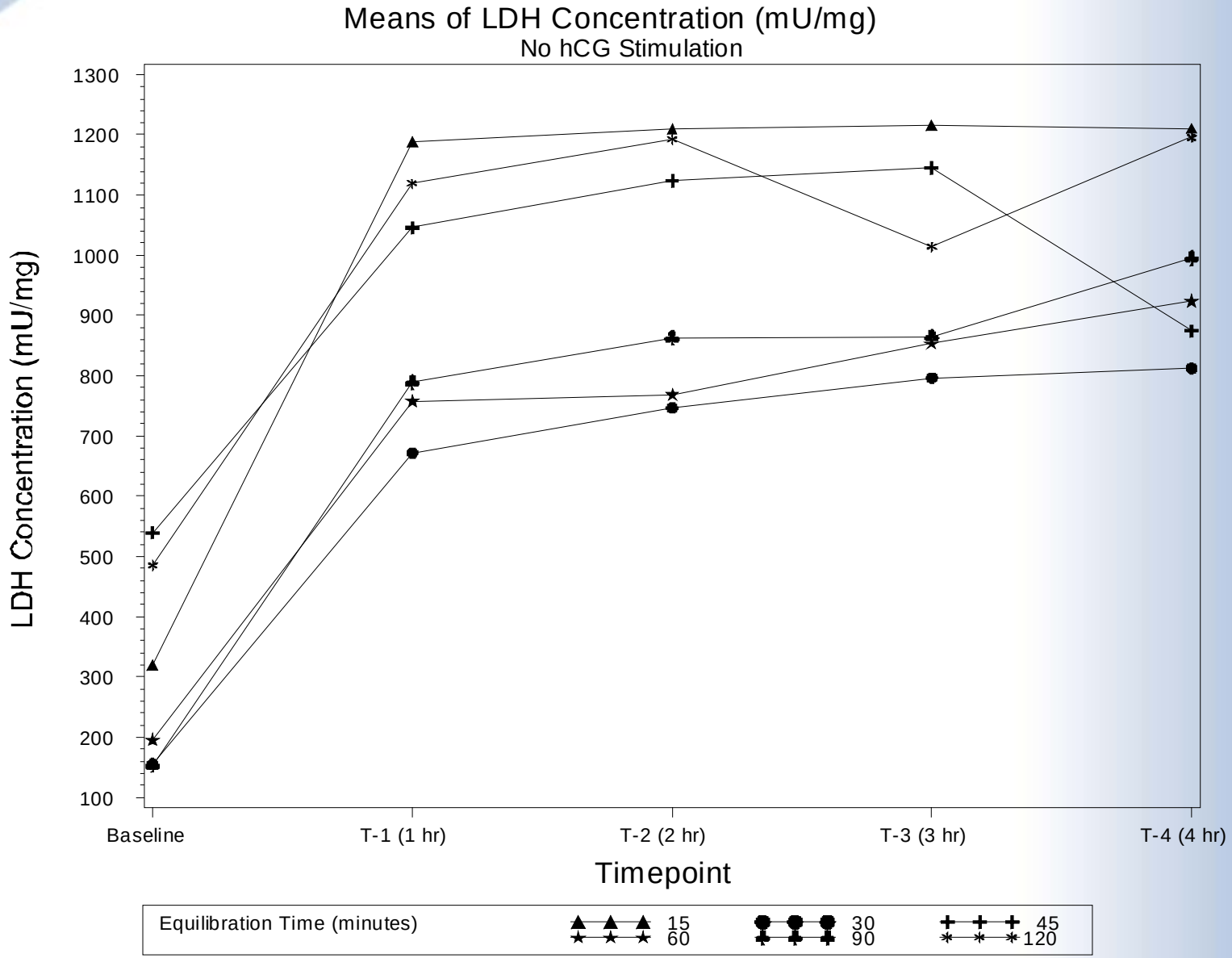

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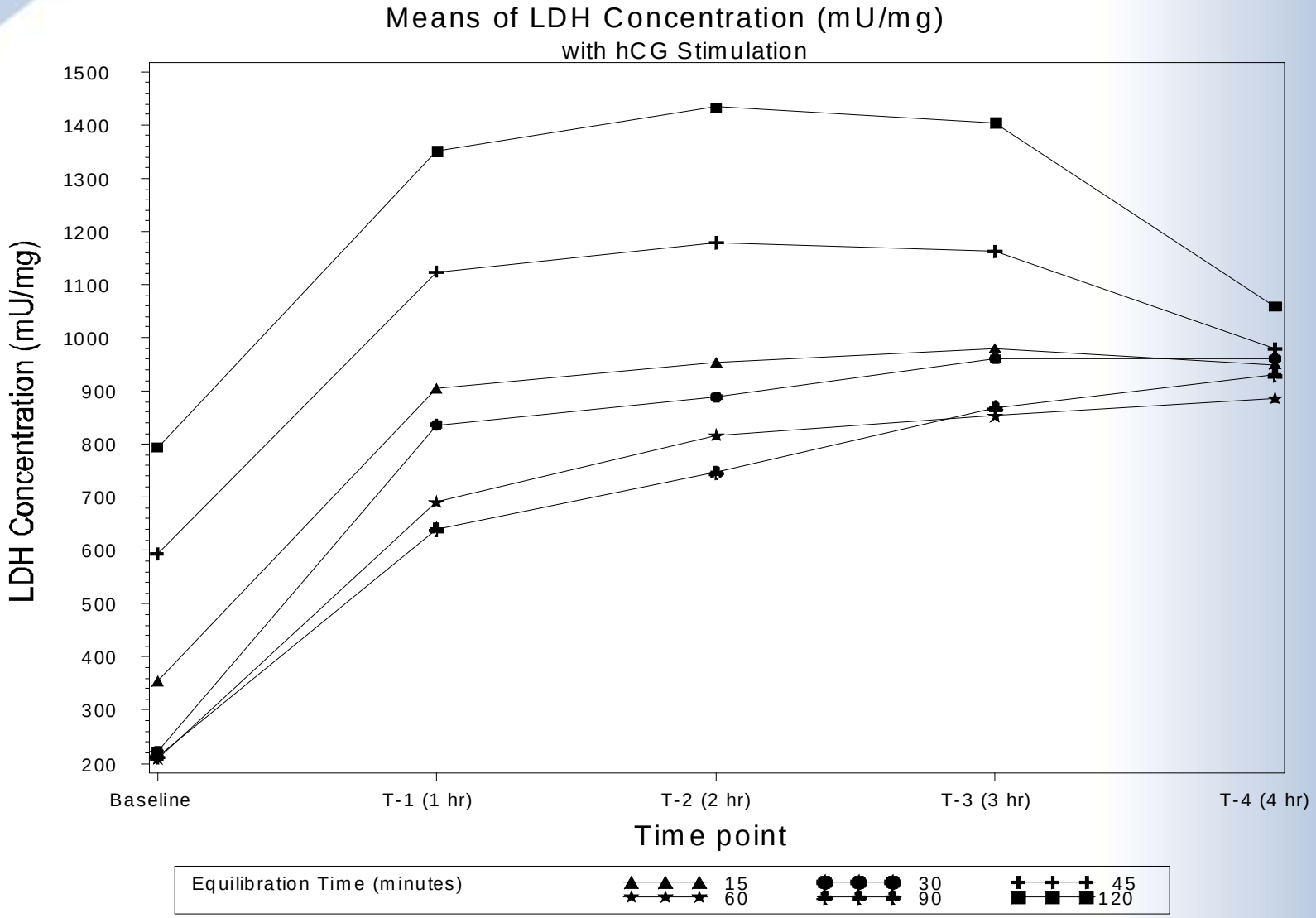

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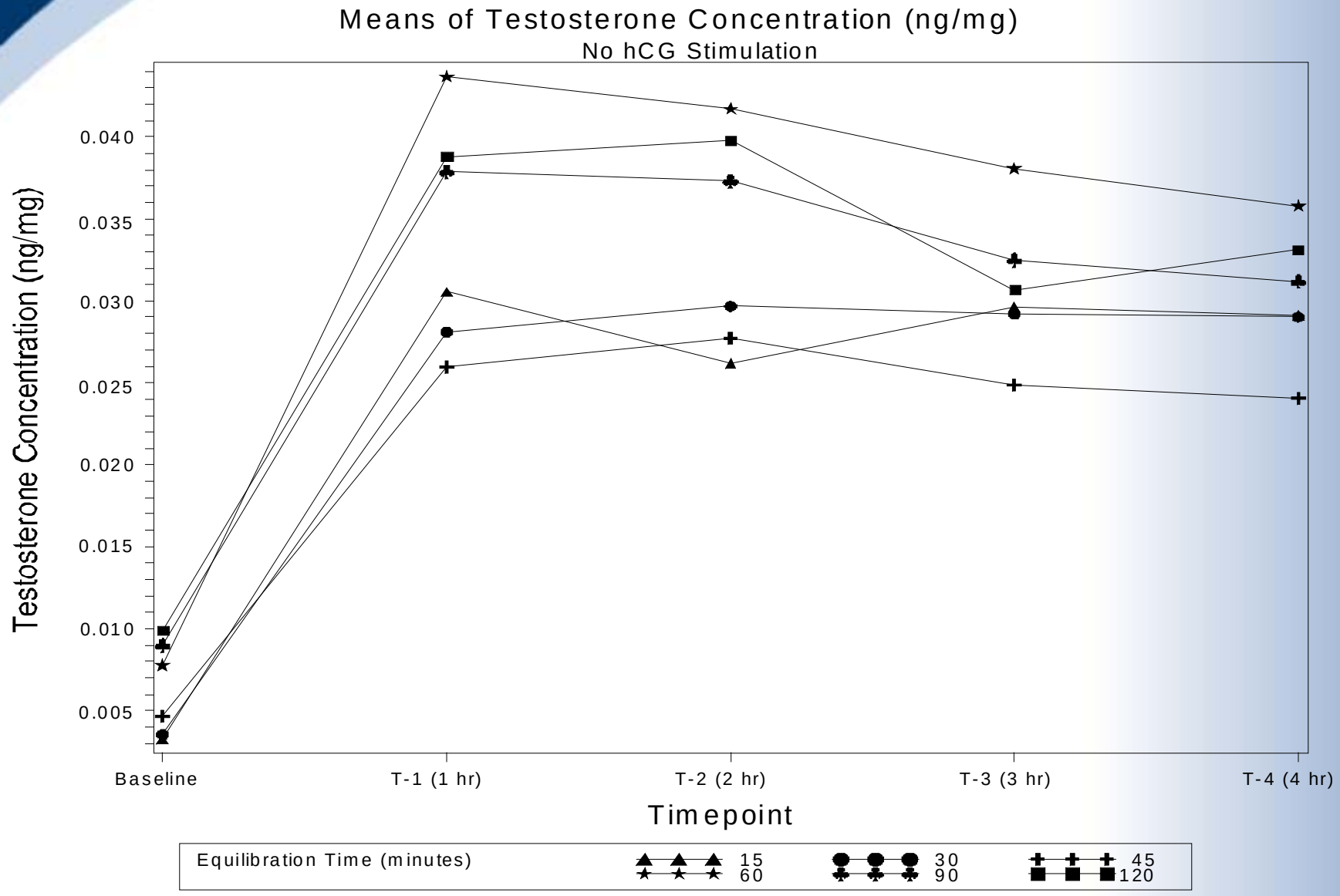

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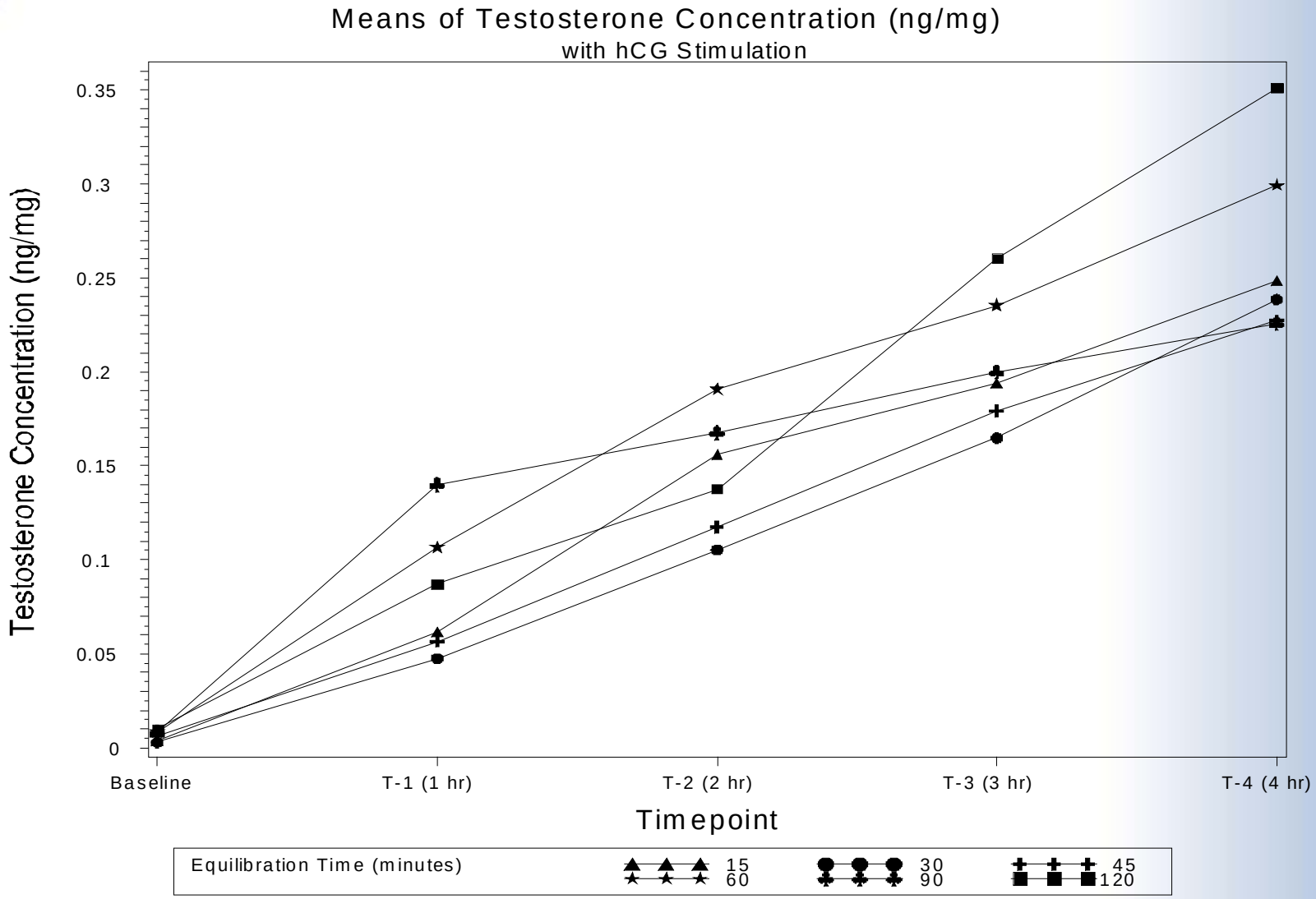

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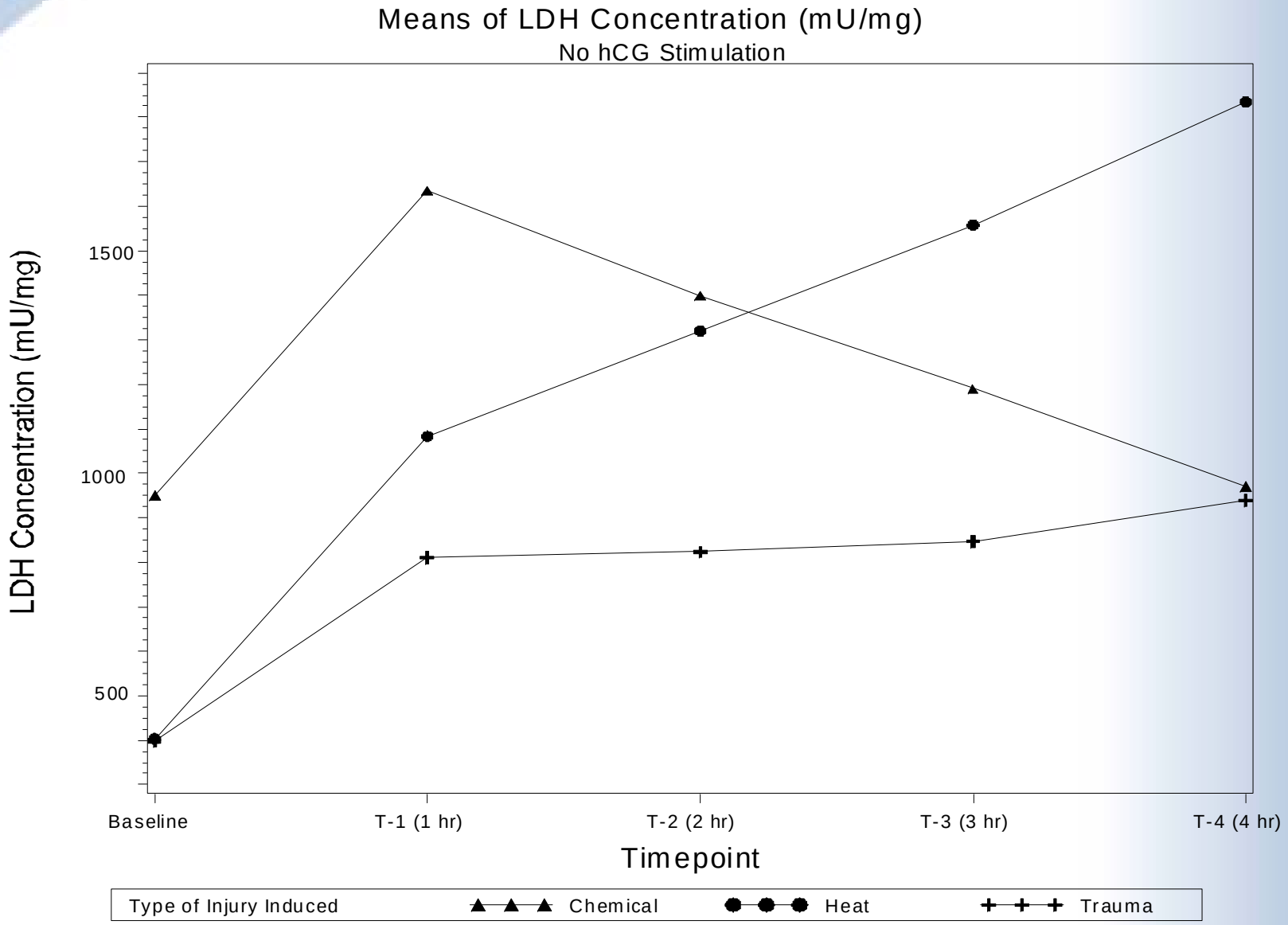
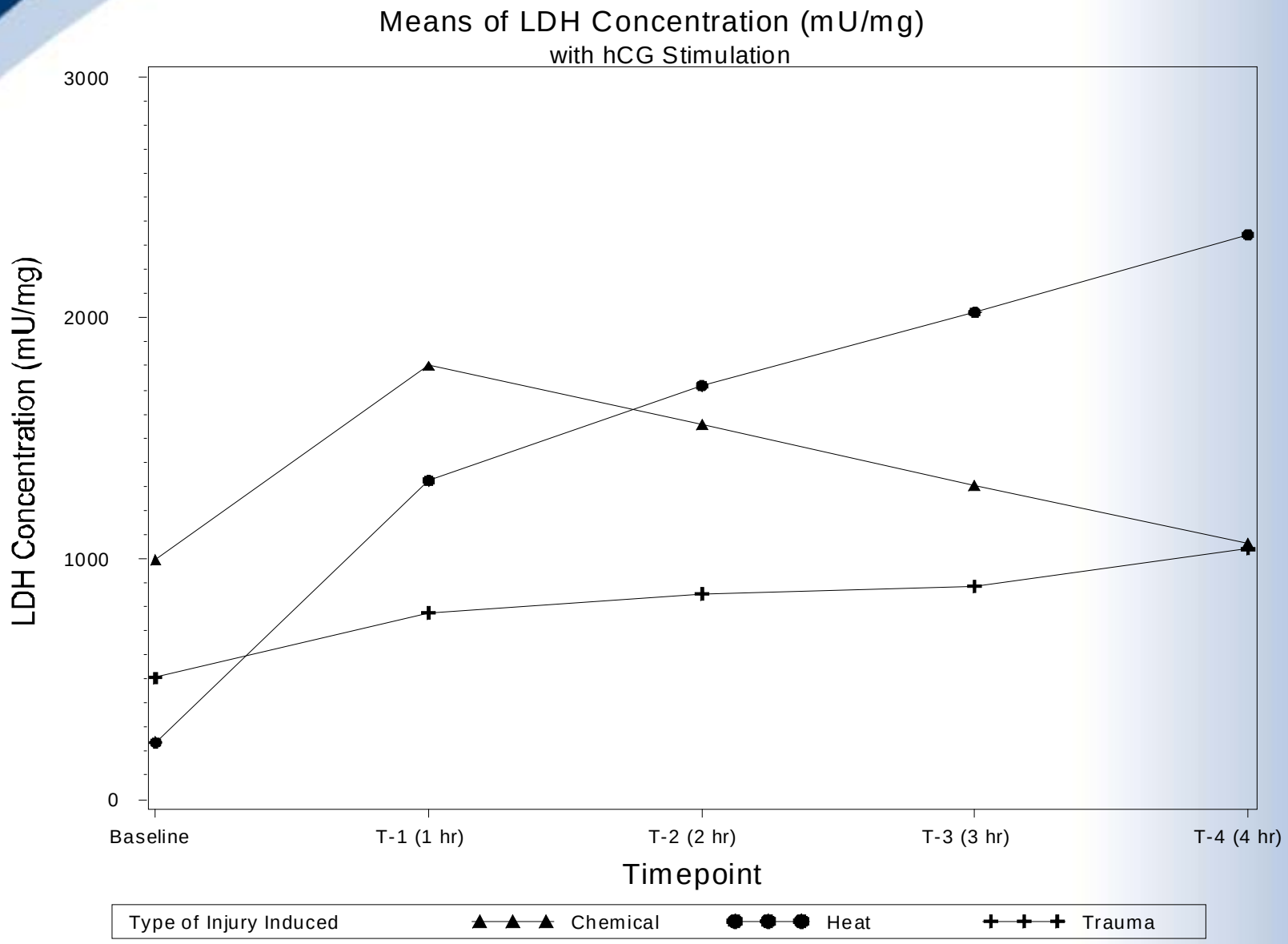
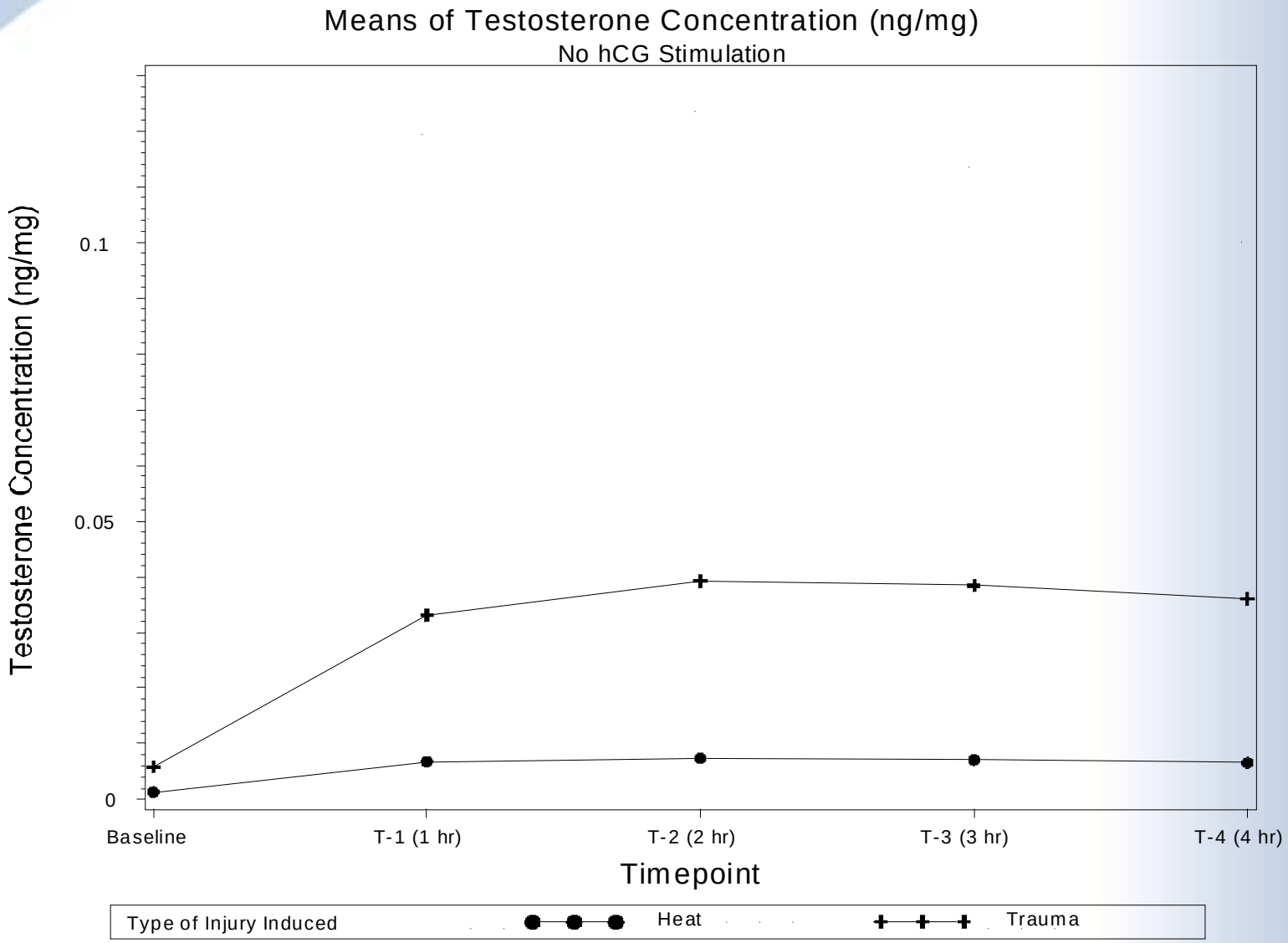
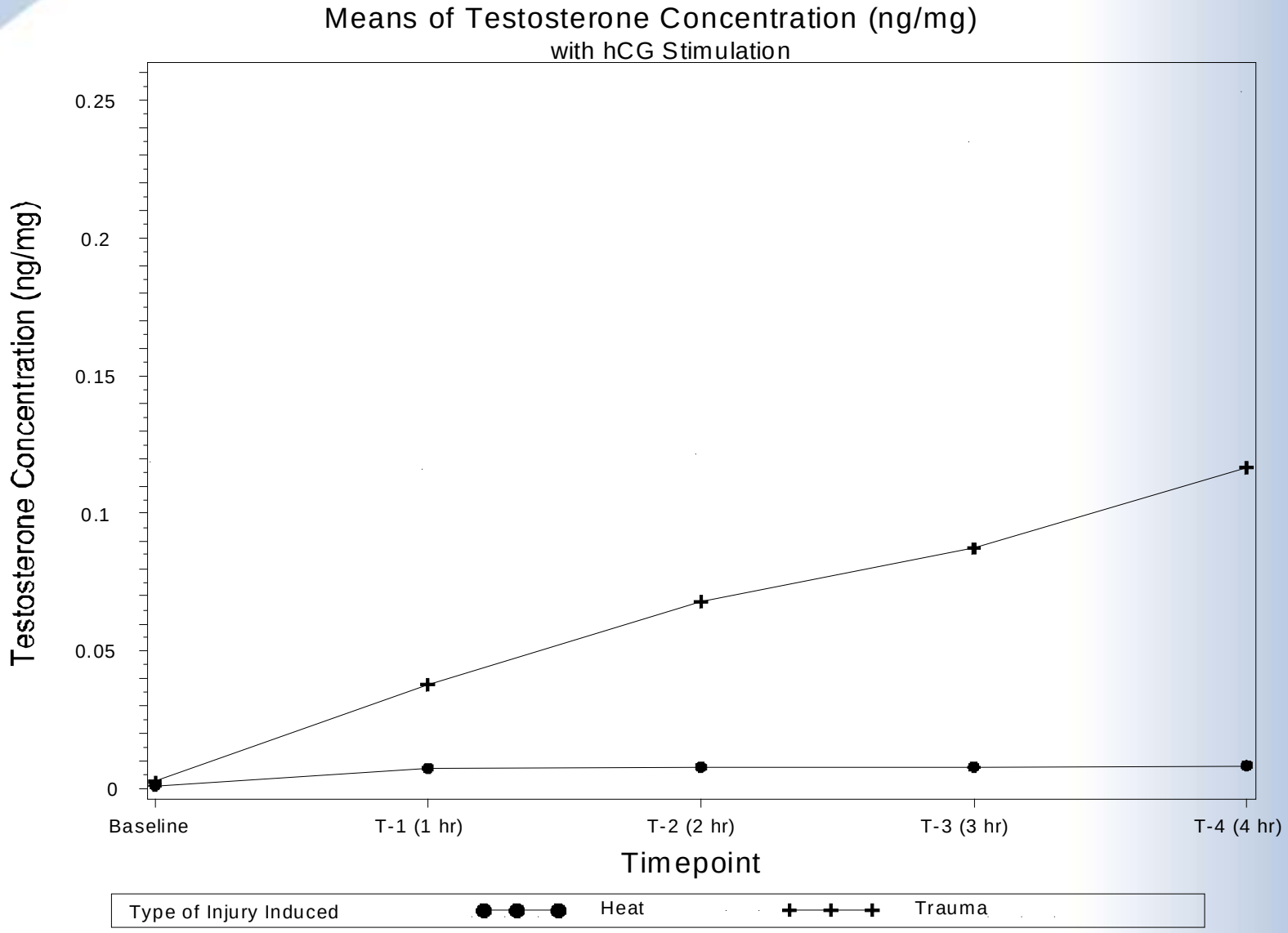

Means of LDH Concentration (mU/mg)
No hCG Stimulation
LDH Concentration (mU/mg)
1500
1000
500
Baseline
T-1 (1 hr)
T-2 (2 hr)
T-3 (3 hr)
T-4 (4 hr)
Timepoint
Type of Injury Induced
Chemical
Heat
Trauma


Means of LDH Concentration (mU/mg)
with hCG Stimulation
LDH Concentration (mU/mg)
3000
2000
1000
0
Baseline
T-1 (1 hr)
T-2 (2 hr)
T-3 (3 hr)
T-4 (4 hr)
Timepoint
Type of Injury Induced
Chemical
Heat
Trauma


Means of Testosterone Concentration (ng/mg)
No hCG Stimulation
Testosterone Concentration (ng/mg)
0.1
0.05
0
Baseline
T-1 (1 hr)
T-2 (2 hr)
T-3 (3 hr)
T-4 (4 hr)
Timepoint
Type of Injury Induced
Heat
Trauma



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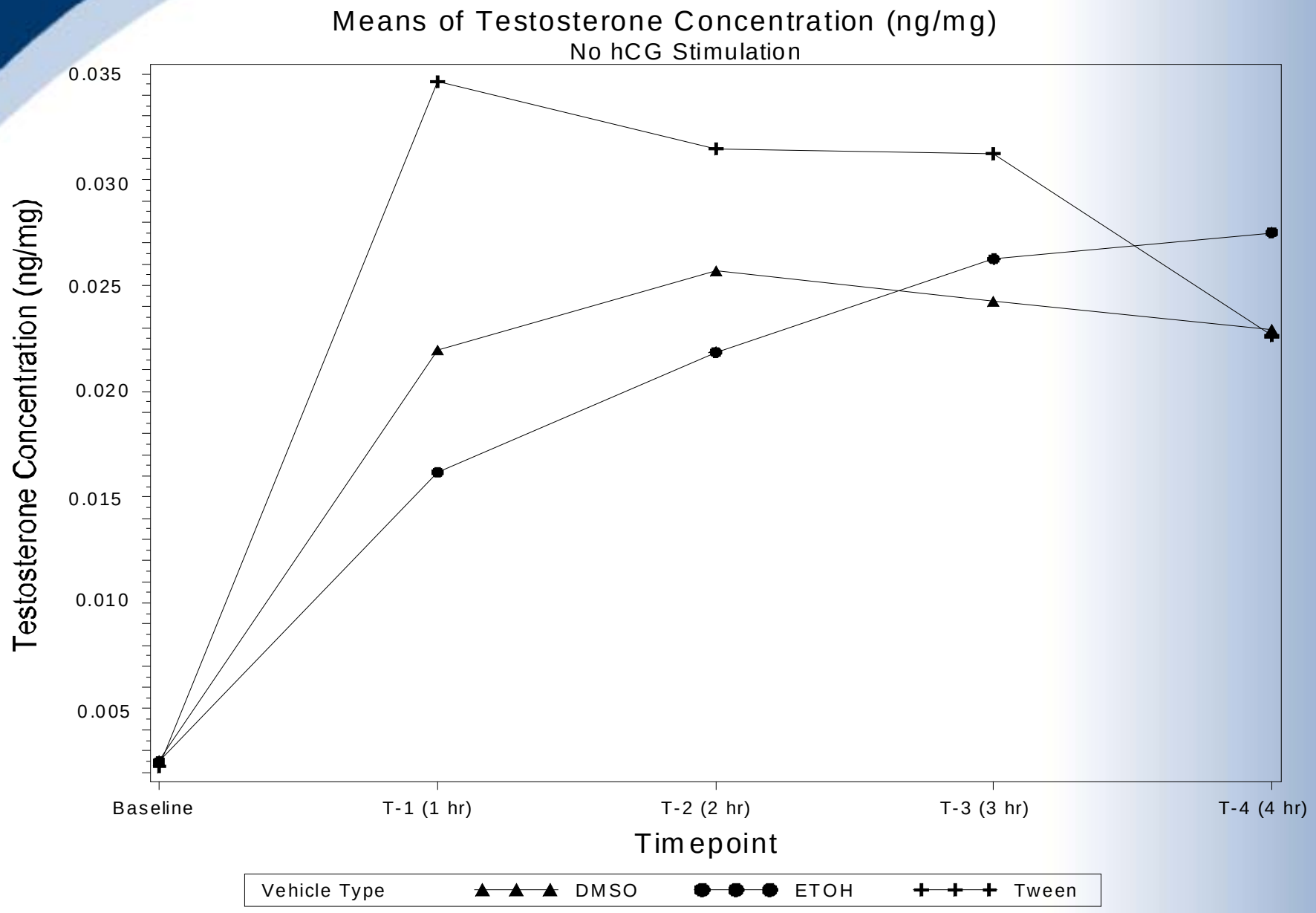

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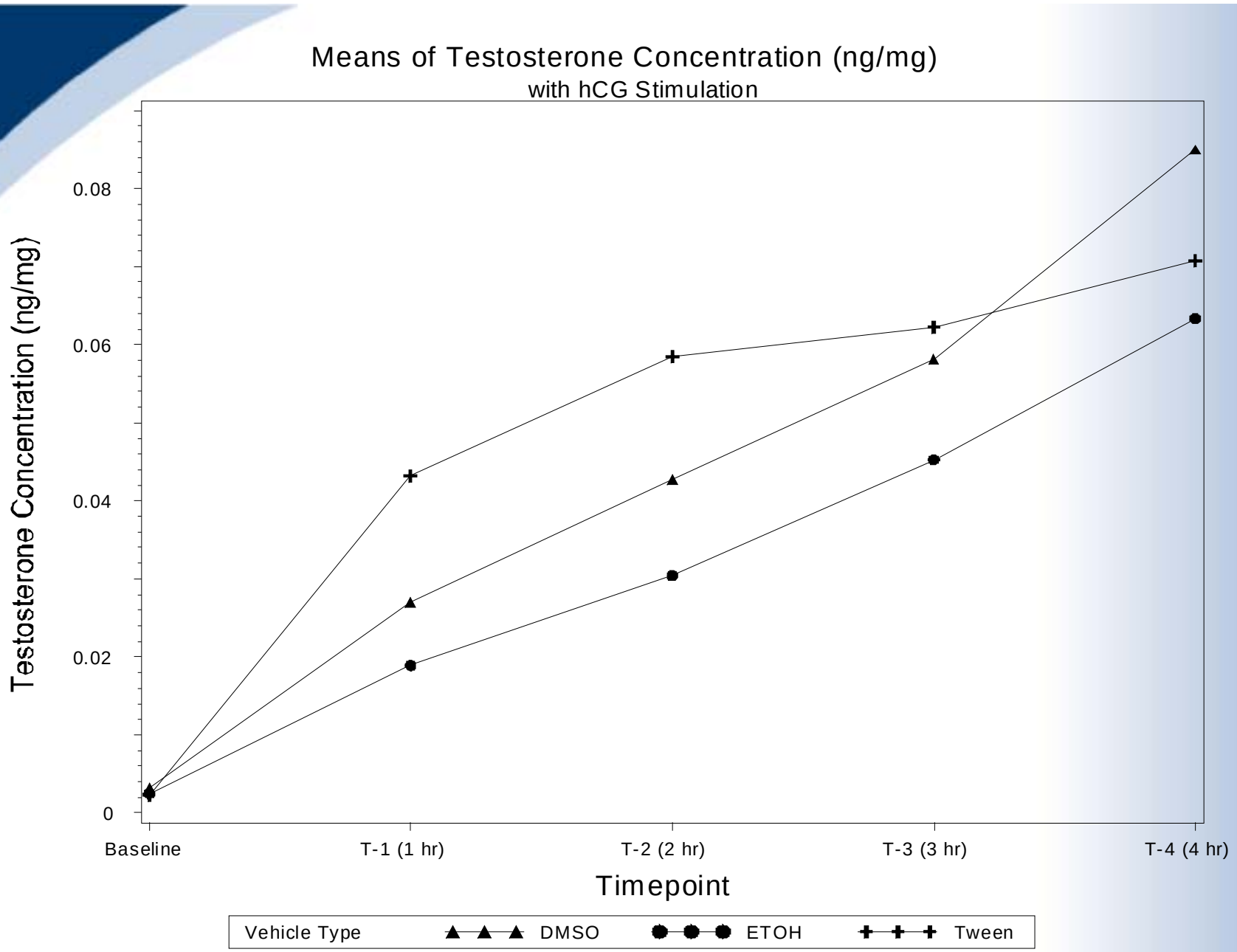

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Summary

- 11-15 week old rats should be used
- Media 199 without phenol red is the preferred media
- Gaseous atmosphere of 5% CO_2 / 95% O_2
- Optimal temperature for incubation was approximately 36ºC
- Optimal vessel for incubation was the scintillation vial
- Optimal Shaker speed was approximately 175 rpm
- Optimal media volume was approximately 4-5 mL
- Optimal hCG concentration for stimulation was 0.08 to 0.1 IU/mL
- Optimal fragment size varied from 100-200 mg
- Time delay before the start of the incubation should be no more than 1 hour from the time of testicular tissue removal
- The solution that the testes are collected in may be either cold DPBS or cold M-199
- The sample aliquot size removed for testing should be around 0.5 mL

Points to Ponder

- All of these assays were performed on control rat testes
- No assays included any test chemicals except for possible vehicles that may be used in the pre-validation and validation assays
- It may be necessary to measure specific cell viability (LDH is not cell specific)
  - Use of beta-HSD (an enzyme specific to Leydig cells) staining

ACKNOWLEDGEMENTS

(RTI International)

- Ms. V. I.. Wilson
- Ms. L. B. Pelletier
- Ms. N. M. Kuney
- Ms. S. W. Pearce
- Ms. K. D. Vick
- Ms. L. McDonald
- Ms. A. J. Parham
- Mr. M. D. Crews
- Ms. A. B. Goodman
- Ms. D. B. Bynum
- Mr. C. Andrew Clayton
- Ms. Margaret Zeller Byron
- Ms. Bonnie Hamby
- Mr. F. N. Ali
- Dr. D. B. Feldman, DVM, ACLAM, Veterinarian

Quality Assurance

- Ms. M. D. Phillips (RTI)
- Mr. R. Patterson, QA Consultant
- Ms. K. Cummings (ILS)

Battelle

- Dr. Jerry D. Johnson
- Dr. David P. Houchens
- Dr. Paul Feder

EPA

- Mr. Gary Timm
- Dr. Jerome Goldman